THE DISTRIBUTION OF CUTIN IN THE OUTER EPIDERMAL WALL OF CLIVIA NOBILIS.

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Epidermal cell walls are commonly considered by botanists as cellulose membranes upon which more or less cutin has been deposited. On the evidence of earlier microchemical work three general regions or zones in epidermal walls are recognized: a thin outer layer of cutin (the cuticle), an inner zone of cellulose and an intermediate zone containing both cutin and cellulose.

The inadequacy of this conception has been demonstrated in brilliant fashion by Frey-Wyssling (2). By studying the effect of the wall upon polarized light Frey-Wyssling has revealed a complexity of structure in certain epidermal walls that was entirely unsuspected. The outer epidermal walls of petioles of *Acuba japonica* were shown to possess curiously isolated plates of cutinized cellulose over each cell. The outer epidermal wall of leaves of *Clivia nobilis* were shown to possess marked differences in double refraction at different levels and a definite zone of isotropic material near the center of the wall, (Fig. 1A).

These researches of Frey led to a further examination of the detailed structure of these epidermal walls from a microchemical point of view. In the case of *Clivia*, the writer (1) was able to detect the presence of three membrane constituents in the wall—cellulose, cutin and pectic materials. The structural relationships between these three membrane constituents is outlined in Fig. 1B. The decrease of double refraction in the cellulose layer Z was related to an increase in amount of colloidal pectic material. The isotropic zone P was caused by the dominance of colloidal pectic material which reached its maximum in this zone. The rapid increase in double refraction as the outer portions of the wall were approached was explained by the decrease in abundance of pectic material in the wall. The maximum double refraction was reached at about the center of the zone of cutinized cellulose and at this point the pectic material reached a minimum. The decrease in double refraction as the cuticle was approached resulted from the steady decrease in the amount of cellulose present until finally when the cuticle C was reached cellulose was no longer present and the wall

became isotropic. The results of the microchemical investigations of this wall suggested an interpretation of the optical results. The two lines of investigation supplemented each other nicely and the results suggest the importance of using both lines of attack in cell wall work.

During the past summer an interesting modification of this epidermal wall was discovered in the leaves of a plant (Clivia) growing in the greenhouse of the Department of Botany of

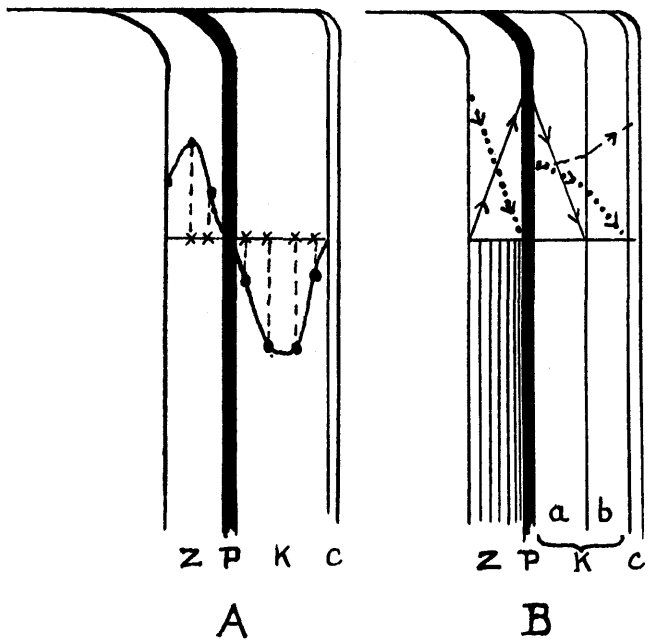

FIG. 1. Diagrammatic representation of the birefringence and chemical relationships in the outer epidermal wall of *Clivia nobilis*.

A. Diagram of the variation in double refraction at different levels in the wall (after Frey). Z represents the cellulose portion adjacent to the cell lumen. Its double refraction reaches a maximum at the center of the layer and drops rapidly as the isotropic zone P is approached. K represents the cutinized cellulose and is optically negative. Double refraction reaches a maximum at the center of the zone and falls rapidly as the outermost layer, C, the cuticle, is approached.

B. Diagram of the relationships of cellulose, pectic material and cutin in the same wall (after Anderson). The cellulose layer Z contains both pectic material and cellulose, the former increasing in amount as the isotropic zone is approached and the latter decreasing. The isotropic zone P contains pectic material with little or no cellulose. The cutinized cellulose K is divided into two zones, one of which (a) contains cellulose, pectic materials and cutin. Pectic materials decrease in amount, cutin increases in amount and cellulose decreases slightly as the zone (b) is approached. Zone (b) contains only cutin and cellulose with the latter decreasing in abundance as the cuticle is approached. The cuticle, C, contains cutin only.

The Ohio State University. Certain leaves of the plant showed a remarkable secondary cutinization of the inner portion of the wall that is apparently unique in epidermal walls. This unusual development is not characteristic of Clivia plants in general yet since it is not only an interesting phenomenon but one that may have an important bearing upon ideas regarding the mechanism of cutinization, the wall was given further study. This paper reports the results of a microchemical investigation of this epidermal cell wall.

METHODS.

Living leaves from the Clivia plant were cut with a sliding microtome into sections 8 or 10 microns in thickness. Similar sections were prepared from leaves preserved in 95% alcohol. These sections were subjected to the usual microchemical tests for membrane substances. Tests of three types were employed, (a) differential staining reactions, (b) differential solubility reactions, and (c) polarized light. The polarizing microscope alone permits the use of three different methods of determining the distribution of certain membrane constituents. Some membrane substances exhibit double refraction while others are isotropic (Plate I, Fig. 7). Insertion of a gypsum plate Red 1 between the nicols permits the use of interference colors to distinguish between some membrane compounds (Plate I, Fig. 8). Advantage may also be taken of dichroism in studing the distribution of lignin or cutin in cellulose membranes.

The various methods serve to check each other. Differential staining is not thoroughly reliable in all cases but is a valuable indicator of the presence or absence of specific materials. Results were not considered conclusive unless indicated independently by each line of attack. The following table gives a brief summary of the principal tests used in this study:

CELLULOSE.

Deep blue color with I_2KI and 65% H_2SO_4.
Blue violet color with chlorzinc iodide.
Unstained with methyl green unless combined with cutin and pectic compounds.
Unstained with Magdala red (echt) unless combined with cutin and pectic compounds.
Unstained with Ruthenium red unless combined with pectic materials.
Soluble in copper oxide ammonia.
Soluble in 50% chromic acid.

Insoluble in hot dilute mineral acids.
Optically positive.
Optically anisotropic.

PECTIC COMPOUNDS.
Deep red color with dilute alkaline Ruthenium red.
Deep blue—blue violet with dilute Methylene blue.
Brown or in some cases colorless with chlorzinc iodide.
Unstained with Methyl green unless combined with cutin and cellulose.
Unstained with Magdala red (echt) unless combined with cutin and cellulose.
Insoluble in copper oxide ammonia.
Soluble in hot dilute mineral acids followed by hot dilute ammonia.
Soluble in 50% chromic acid.
Optically isotropic.

CUTIN.
Red color with Sudan III.
Red color with Scharlack R.
Violet stain with Methyl green when combined with cellulose and pectic materials.
Colorless with Methyl green when free from cellulose and pectic materials.
Red color with Magdala red (echt) when combined with cellulose and pectic compounds.
Colorless with Magdala red (echt) when free from cellulose and pectic compounds.
Brown color with chlorzinc iodide.
Insoluble in 50% chromic acid, copper oxide ammonia, and hot dilute mineral acids.
Saponified with hot concentrated KOH.
Optically negative when combined with cellulose.
Optically anisotropic when combined with cellulose.
Optically anisotropic or optically isotropic when free from cellulose.

Mention should be made of the importance of Methyl Green and Magdala Red introduced by Kisser (4) as a means of detecting cutinized cellulose. Methyl Green commonly contains small quantities of Methyl Violet as an impurity. This Methyl Violet is strongly absorbed by cutinized cellulose containing pectic compounds from dilute solutions of Methyl Green. Beautiful results may be obtained with the dye if properly used and it can be most instructive in determining the presence of combinations of the three membrane constituents mentioned. The intensity of the stain seems proportional to the abundance of cellulose and pectic materials. Magdala Red works equally well, giving a distinct red color that sharply differentiates the different regions in the wall.

These dyes are also helpful in studying lignification as Kisser has pointed out.

Through the courtesy of Professor W. Seifriz the writer was able to examine preparations of the wall with the Spierer lens. The image gave the characteristic striated appearance reported by Seifriz (6) and others in the cellulose and outer cutinized portions of the wall. The inner zone of cutinized cellulose presented an entirely different aspect. In place of the symmetrically arranged striations the dark rods were chaotically distributed, giving the appearance of much folding and irregularity of arrangement. This indicated a different physical relationship in the inner zone of cutinized cellulose over that prevailing in the outer portions of the wall.

In addition to the interference colors obtained with a gypsum plate Red I, the polarizing microscope permits the use of dichroism as a tool in cell wall work. Ambronn discovered this phenomenon in 1888 but it was the work of Frey-Wyssling (3) that has demonstrated the importance of the phenomenon in cell wall work. Dichroism is apparently the result of the micellar structure of the cellulose wall. When a bast fiber of flax (Linum) is stained with chlorzinc iodide and examined with a polarizing microscope, using the polarizer only, the color of the fiber varies from almost black to colorless when the stage is rotated. The following quotation from Frey-Wyssling indicates the way in which this phenomenon may be utilized in studying the distribution of cutin in epidermal walls:

"Die gelben Reaktionen von Lignin, Suberin und Kutin sind im Gegensatz zur Zellulosereaktion nicht dichroitisch. Frisch gefärbte Schnitte von Holz- und Korkgeweben, sowie stark kutinisierte Epidermen (z. B. von *Clivia nobilis*) weisen keine Intensitätsunterschiede auf, wenn man den Objekttisch über dem Polarisator dreht.

"Diese Feststellung ist wichtig; denn sie gestattet uns den Nachweis von Lignin und Kutin in nur schwach imprägnierten Zellmembranen, die mit vielen Reagentien nur Zellulosereaktionen geben. Färbt man solche Objekte, wie z. B. Jutefasern oder die langen Haare des Fruchtschnabels *Erodium gruinum*, mit Chlorzinkjod und bringt sie über dem Polarisator in die Stellung 'farblos,' so erscheinen sie je nach dem Ligningehalt schwächer oder stärker gelb gefärbt.

"So erlaubt uns das Chlorzinkjod nicht nur völlig verholzte und kutinisierte Membranen zu erkennen, sondern mit Hilfe

des Dichroismus der Zellulosereaktion können in der Stellung des Absorptionsminimums selbst die ersten Anfänge der chemischen Zellwandveränderungen, die oft schwer nachzuweisen sind, spielend enthüllt werden."

All of the above mentioned methods have been used in studying this epidermal wall—and results from the various methods are in agreement.

RESULTS.

Staining the wall with Sudan III brings out clearly the two distinct zones of cutinization (Plate I, Fig. 1). The outer zone and the inner zone of the cutinized wall are distinctly separated by a colorless zone of cellulose and pectic material. The structure of this deposit has been reported in an earlier paper (1). In some cases the inner cutinized zone is adjacent to the protoplasm of the epidermal cell while in other cases a second zone of cellulose separates the protoplast from the inner zone of the cutinized wall. Both conditions may be seen in the figure cited. Careful staining with dilute Sudan III reveals a difference in intensity of stain in the outer portion of the wall. The same effect may be produced by staining with Sudan III and then carefully washing out the stain with alcohol. The vertical boundary line between each cell is emphasized by a deeply staining, vertical, rod-shaped region (Plate I, Fig. 2). This zone differs decidedly from the rest of the cutinized wall in its higher content of cutin, and in the ease with which it may be saponified. The cellulose in the outer wall is not continuous, but grouped in a series of fine horizontal lamellae over each cell. Between these groups of cellulose lamellae extends this narrow vertical partition of nearly pure cutin, partially isolating each cell from other cells in the outer wall. This structure is clearly revealed when the wall is saponified with concentrated KOH (Plate I, Figs. 5 and 6). The presence of this vertical partition of cutin between the epidermal cells is further revealed in striking fashion by the polarizing microscope (Plate I, Figs. 7 and 8). The outermost layer of cutin, the cuticle, is more resistant to saponification and remains as a definite sheet-like layer when the cutin has been removed from the remaining portions of the wall with saponifying agents (Plate I, Figs. 5 and 6).

The presence of cutin in the inner cutinized zone is not only indicated by the adsorption of Sudan III, but also by the action

of concentrated chromic acid. This reagent quickly removes the cellulose but aside from a slight swelling that reveals a distinct lamellation, the inner zone of cutin is unaffected (Plate I, Fig. 4). Apparently this inner cutinized zone consists of a group of parallel lamellae of cellulose, separated by pectic material and infiltrated with cutin. Saponification of the cutin in this zone produces globules of soap as readily as the cutin present in the outer portion of the wall (Plate I, Fig. 5). The lamellae that are visible after saponification give the recognized tests for cellulose. The inner zone shows the interference colors characteristic of cutinized cellulose, though the colors are less conspicuous than those of the outer portions of the wall (Plate I, Fig. 8). The high pectic content of the inner cutinized zone is indicated by the intensity of the staining reactions (Magdala Red, Methyl Green), by the solubility reactions and by the marked decrease in double refraction of this portion of the wall (Plate I, Fig. 7). The inner cutinized zone exhibits a slight but definite double refraction and gives the dichroism characteristic of cutinized cellulose when treated with chlorzinc iodide.

DISCUSSION.

The outer epidermal wall of the leaves of the *Clivia nobilis* plant studied, differs from the outer epidermal wall of most Clivia plants in having two definite zones of cutinized cellulose. The cellulose deposited by the protoplasm of the epidermal cell is unevenly distributed around the vacuole, being much thicker on the outer wall. This cellulose deposit is not homogeneous, but consists of a series of fine horizontal lamellae separated by colloidal pectic material. The amount of pectic material varies in different portions of this outer wall and the present investigation confirms the structure reported in an earlier paper in this respect. This complex of cellulose and pectic lamellae is deposited only above each protoplast and does not extend continuously from cell to cell. The apparent continuity of the epidermal wall is due to the presence of cutin that impregnates the stratified deposition of each cell and fills the spaces between the cells, welding them into one wall. The outer epidermal wall is therefore essentially a mosaic of the cutinized cellulose of individual cells bound together by vertical partitions of cutin.

The inner zone of cutinized cellulose has in general the same

fundamental structure present in the outer portion of the wall. The deposit consists of a series of thin cellulose lamellae, separated by and impregnated with colloidal pectic material. To this has been added the cutin. The thickness of this inner cutinized zone varies widely in different cells of the same section and is in general thicker in the upper epidermis than in the lower. In some cells the deposit is apparently in direct contact with the living protoplast while in others it is separated from the protoplast by a second deposit of cellulose.

Concerning the cause for this unique deposit little can be said. It is not general and the author has not seen it in his earlier work with Clivia plants. It seems limited to relatively few individuals through an examination of the leaves of Clivia plants in various parts of the country may reveal that the habit is more general than realized at present. The deposit is of particular interest in regard to present theories of the mechanism of cutinization. Lee and Priestley (5) have suggested that the fatty constituents of epidermal walls may reach the epidermis by migration along the radial walls of the subepidermal tissues. On reaching the outer wall the fatty materials are condensed and oxidized forming the cutinized portions of the epidermal wall. The present study offers support to their suggestion that exposure to the light and air modifies considerably the character of the fatty deposits for the outermost layer is decidedly more resistant to saponification than are the deposits of cutin within the wall itself. It is difficult to account for this inner localized zone of cutin completely surrounded by cellulose, or at least bordered by cellulose on the *outer* margin on the assumption that the fatty constituents were migrating to the surface through the walls of the subepidermal cells and deposited in the walls through the loss of water.

In this case the epidermal wall seems to be produced by the epidermal cells themselves, and furthermore it indicates that the mechanism of cutinization may be more complex than generally realized at present. It is not impossible that the cutin partitions between the cutinized cellulose of each epidermal cell may arise from subepidermal tissue and reach the epidermis by a migration, and it may be that some of the cutin in the outer wall has a similar origin. The general structure of the wall and the presence of these peculiar localized zones of cutin in the wall do not support this suggestion. It seems probable that in this wall the cutin as well as the cellulose and pectic

materials are all the result of the activities of the epidermal cells alone.

The inner cutinized zone is usually characterized by the appearance of numerous fine granules of fatty material. The delicate cellulose lamellae seem at times to be strings upon which cutinized beads are strung. The cutin does not have the smooth uniformity of that in the outer portions of the wall. These granules may be seen in Figs. 1, 3, and 4 of the plate. The Spierer lens further indicates that the arrangement of the units composing the wall are not regular nor uniform in this inner cutinized zone. The significance of these fine granules is unknown. In some cases gaps and spaces are evident in the inner cutinized zone. These may have resulted from sectioning but seem to indicate that this portion of the wall has less cohesion than other portions.

A large part of the experimental work described in this paper was accomplished in the Plant Microchemical Laboratories of The Ohio State University and the author wishes to express his appreciation of the many courtesies extended to him by members of the Botany Department of that institution. The author further wishes to acknowledge his indebtedness to Professor Seifriz, of the University of Pennsylvania, for the opportunity of examining sections of this wall with the Spierer Lens and to thank Dozent Dr. Frey-Wyssling, of Zürich, for his courtesy in checking dichroic and interference phenomena of the wall.

SUMMARY.

1. The epidermal cell walls of the leaves of some *Clivia nobilis* plants show two distinct zones of cutinization.

2. The inner zone of cutinized wall consists of a series of cellulose lamellae separated by layers of pectic material, both of which are impregnated with cutin.

3. The inner cutinized zone may be in direct contact with the protoplasm of the cell or may be separated from the protoplasm by a second zone of cellulose and pectic materials.

4. The inner cutinized zone is separated from the outer cutinized zone by cellulose and pectic material. The structure of this deposit has been reported in an earlier paper.

5. The cutin is not uniformly deposited in the outer cutinized zone, nor has it the same chemical and physical properties in all portions of the zone.

6. The outermost layer of the wall contains no cellulose

nor pectic material and is more resistant to saponification than other cutinized areas of the wall.

7. The cellulose and pectic material in the outer wall of each epidermal cell are separated from other cells by vertical partitions of cutin that contain little or no cellulose.

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EXPLANATION OF PLATE I.

(All figures refer to cross sections of epidermal cells of leaves of *Clivia nobilis*.)

Fig. 1. Wall stained with Sudan III. Cutinized regions black, cellulose and pectic materials free from cutin are white. 440×

Fig. 2. Wall stained with Sudan III and partially destained with alcohol, showing vertical partitions of cutin between the outer wall of each cell. 440×.

Fig. 3. Wall stained with Magdala Red (echt). Inner zone of cutin, pectic materials and cellulose stains, vertical cutin partitions revealed and accumulation of pectic material indicated at boundary between cellulose and outer zone of cutinized cellulose. 440×.

Fig. 4. Wall treated with 50% Chromic acid. Cellulose largely removed. Inner zone of cutinized cellulose swollen to reveal lamellated structure. 440×.

Fig. 5. Wall treated with hot conc. KOH, followed after washing, with Chlorzinc iodide. Resistant cuticle remains. Vertical cutin partitions between cells removed, cellulose lamellae in outer walls stained blue. Inner zone of cutinized cellulose showing lamellations and soap globules. 440×.

Fig. 6. Wall treated with hot conc. KOH. Vertical partition of cutin completely removed. Soap globules apparent in zone of cutinized cellulose. Cuticle intact showing high resistance to saponification. Outer portion of wall only is shown. 440×.

Fig. 7. Wall as seen between crossed Nicols. Pectic zone in the wall appears as a black line. High pectic content of the inner cutinized zone markedly reduces its double refraction. 500×.

Fig. 8. Wall as seen between crossed Nicols with gypsum plate Red I inserted showing Interference colors. Vertical cutin partitions conspicuous. Inner cutinized zone optically negative indicating presence of cutin. 500×.

PLATE 1.